FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

# TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: LASP:111_US_

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5): 09/857960

| INTERNATIONAL APPLICATION NO. | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| PCT/DE99/03946 | December 10, 1999 | December 17, 1998 |

TITLE OF INVENTION

**METHOD FOR DIFFERENTIATED INVESTIGATION OF DIVERSE STRUCTURES IN PREFERABLY BIOLOGICAL PREPARATIONS**

APPLICANT(S) FOR DO/EO/US

**Johann ENGELHARDT**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

**Items 13 to 20 below concern document(s) or information included:**

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☒ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

**Acknowledgement postcard**
**Assignee for purposes of publication: Leica Microsystems Heidelberg GmbH Mannheim, Germany**

| U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5) | INTERNATIONAL APPLICATION NO. | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| 09/857960 | PCT/DE99/03946 | LASP:111_US_ |

24. The following fees are submitted:.

**BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :**

| | | CALCULATIONS PTO USE ONLY |
|---|---|---|
| ☐ | Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . . | **$1000.00** |
| ☒ | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . . . | **$860.00** |
| ☐ | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . . | **$710.00** |
| ☐ | International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . . | **$690.00** |
| ☐ | International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . . | **$100.00** |

| | | |
|---|---|---|
| **ENTER APPROPRIATE BASIC FEE AMOUNT =** | **$860.00** | |
| Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). | **$0.00** | |

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
|---|---|---|---|---|---|
| Total claims | 23 - 20 = | 3 | x $18.00 | **$54.00** | |
| Independent claims | 1 - 3 = | 0 | x $80.00 | **$0.00** | |
| Multiple Dependent Claims **(check if applicable).** | | | ☐ | **$0.00** | |
| **TOTAL OF ABOVE CALCULATIONS =** | | | | **$914.00** | |
| ☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2. | | | | **$0.00** | |
| **SUBTOTAL =** | | | | **$914.00** | |
| Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). + | | | | **$0.00** | |
| **TOTAL NATIONAL FEE =** | | | | **$914.00** | |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☒ | | | | **$40.00** | |
| **TOTAL FEES ENCLOSED =** | | | | **$954.00** | |
| | | | | **Amount to be: refunded** | $ |
| | | | | **charged** | $ |

a. ☒ A check in the amount of **$954.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0822** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

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24041
PATENT_TRADEMARK OFFICE

SIGNATURE

**George L. Snyder, Jr.**
NAME

**37,729**
REGISTRATION NUMBER

**June 12, 2001**
DATE

Express Mail Label No. EL917291267US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**
**PATENT COOPERATION TREATY**

| | | |
|---|---|---|
| Applicant(s): | Johann ENGELHARDT | Atty Ref: LASP:111_US_ |
| Serial No.: | unknown | Group Art Unit: unknown |
| Filing Date: | unknown | Examiner: unknown |
| Title: | METHOD FOR DIFFERENTIATED INVESTIGATION OF DIVERSE STRUCTURES IN PREFERABLY BIOLOGICAL PREPARATIONS | |

International Application No.: PCT/DE99/03946

International Filing Date: December 10, 1999

PRELIMINARY AMENDMENT

Box PCT
Commissioner for Patents
Washington, D.C. 20231

Honorable Sir:

Please preliminarily amend the above-identified application concurrently filed under 35 USC 371 as follows:

*IN THE SPECIFICATION:*

Please enter the enclosed Substitute Specification, which is provided herewith in both a Marked Version showing changes from the English translation of the International Application and an Unmarked Version. The Substitute Specification contains no new matter.

*IN THE CLAIMS:*

Please cancel claims 1-31 and add the following new claims 32-54:

32. (new) A method for the differentiated examination of various structures in a biological preparation using a microscope, said method comprising the steps of:

A) assigning particles with a specific diameter and specific characteristics to said structures; and

B) detecting said structures by detecting said particles specifically bound in or on said structures of said preparation using light that acts on said particles, said particles possessing constant characteristics independent of the time of irradiation by said light.

33. (new) The method as recited in Claim 32, wherein said particles are detected by selecting a wavelength of suitable light being as a function of said diameter and of said specific characteristics of the particles such that said particles are detected on the basis of a Mie scatter occurring on said particles.

34. (new) The method as recited in Claim 32, wherein said particles are detected by selecting a wavelength of a suitable light as a function of said diameter and of said specific characteristics of said particles such that said particles are detected on the basis of a plasmon signal occurring on said particles.

35. (new) The method as recited in Claim 33, wherein said wavelength of said light is larger than, or is approximately equal to, said diameter of said particles.

36. (new) The method as recited in Claim 32, wherein areas of said preparation to be differentiated are provided with particles of various diameters, so that said areas to be differentiated are detected simultaneously or successively by means of suitable light of various wavelengths.

37. (new) The method as recited in Claim 32, wherein said particles are metallic particles or particles metalized on the surface.

38. (new) The method as recited in Claim 37, wherein said particles are formed as ellipsoids or beads.

39. (new) The method as recited in Claim 33, wherein said particles are detected through the Mie-reflexes occurring there in transmission microscope mode.

40. (new) The method as recited in Claim 39, wherein said microscope is a conventional polarization transmission microscope or a confocal polarization transmission microscope.

41. (new) The method as recited in Claim 33, wherein the specific detection of the particles is achieved via the Mie-reflexes occurring there in the reflection microscope mode.

42. (new) The method as recited in Claim 10, wherein said microscope is a conventional polarization reflection microscope or a confocal polarization reflection microscope.

43. (new) The method as recited in Claim 32, wherein said light is produced using a high-pressure lamp as a light source.

44. (new) The method as recited in claim 43, wherein said light source comprises means for wavelength selection and polarization.

45. (new) The method as recited in Claim 32, wherein said light is produced using a laser as a light source, said laser emitting polarized light of one wavelength.

46. (new) The method as recited in Claim 32, wherein said light is produced using an optical parametric oscillator as a light source, the wavelength of said light being variable using said optical parametric oscillator, whereby a maximum Mie-signal for a specific particle type can be measured.

47. (new) The method as recited in Claim 32, wherein said light is produced using a laser as a light source, said laser emitting polarized light of several different wavelengths, and means for selecting wavelengths is connected in series to said laser.

48. (new) The method as recited in Claim 47, wherein said means for selecting wavelengths is integrally connected in to said laser.

49. (new) The method as recited in Claim 47, wherein said means for selecting wavelengths is integrally connected in to said laser.

50. (new) The method as recited in Claim 32, further comprising the steps of:

C) recording a detection image and a conventional transmitted light microscopic image using said microscope; and

D) evaluating said recorded images using digital image processing;

whereby said biological preparation is analyzed.

51. (new) The method as recited in Claim 32, further comprising the steps of:

C) recording a detection image and a conventional reflected light microscopic image using said microscope; and

D) evaluating said recorded images using digital image processing;

whereby said biological preparation is analyzed.

52. (new) The method as recited in Claim 32, further comprising the steps of:

C) recording a detection image, a conventional transmitted light microscopic image, and a conventional reflected light microscopic image using said microscope; and

D) evaluating said recorded images using digital image processing;

whereby said biological preparation is analyzed.

53. (new) The method as recited in Claim 32, further comprising the steps of:

C) recording a plurality of images under various lighting and detection angles; and

D) evaluating said plurality of recorded images using digital image processing;

whereby said biological preparation is analyzed.

54. (new) The method as recited in Claim 32, wherein said particles are coated on the surface and the coating enables a specific bonding to corresponding complementary structures of said preparation.



REMARKS

Entry of the Substitute Specification and new claims 32-54 is respectfully requested. If the examiner has any questions, he or she may contact the undersigned attorney at the number provided below.

Respectfully submitted,

SIMPSON, SIMPSON & SNYDER, L.L.P.

By _____
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Dated: June 12, 2001

Express Mail Label No. EL 917291267 US LASP:111_US_

Substitute Specification - Unmarked Version

# TITLE OF THE INVENTION

Method for the Differentiated Examination of Various Structures in Preferably Biological Preparations

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** The present application is the U.S. national phase under 35 U.S.C. 371 of International Application No. PCT/DE99/03946 filed December 10, 1999 claiming priority of German Patent Application No. 198 58 431.8 filed December 17, 1998 and German Patent Application No. 199 50 909.3 filed October 22, 1999.

## FIELD OF THE INVENTION

**[0002]** The invention relates to a method for the differentiated examination of various structures in preferably biological preparations, in particular via confocal laser microscopy.

## BACKGROUND OF THE INVENTION

**[0003]** Fundamentally, this involves a detection/marking method, in particular a method as is applied within the context conventional fluorescence microscopy within the biomedical field. However, the fluorescence microscopy previously employed is in practice exceptionally problematic, particularly since the fluorescent dies used therein fade over time, and specifically have a fading characteristic that prevents the reproduction of examinations. Because of this fading characteristic, the fluorescence intensities change even during the course of microexamination and in particular when there is radiation of the fluorescent die with excitation light. This not only makes a reproduction of the examination impossible, but, what is more, it also makes any examination subsequent to radiation of the biological/medical preparation more difficult or – in terms of a reliable evaluation – makes such an examination nearly impossible.

## BRIEF SUMMARY OF THE INVENTION

**[0004]** The objective of the present invention is therefore to configure a method of the type in question for differentiated examination of various structures in preferably biological preparations, in particular via confocal laser scanning microscopy, such that the reproduction of the marking or of the result of the examination is ensured, even after prolonged radiation. The problems occurring in fluorescence microscopy or in connection with fluorescent die binding are to be thereby prevented.

**[0005]** This objective is met via a method for the differentiated examination of various structures in preferably biological preparations, in particular using a confocal laser scanning microscope, as described and claimed herein. Accordingly, the method for differentiated examination of various structures in preferably biological preparations is characterized in that particles with a specific diameter and specific characteristics are assigned to the structures, and said structures are detected by detection of the particles specifically bound in or on the preparations.

**[0006]** In an advantageous manner the particles are detected by virtue of the wavelength of the appropriate light being selected as a function of the diameter and the specific characteristics of the particles such that the particles can be detected on the basis of the Mie scatter or Mie reflexes occurring on the particles.

**[0007]** Alternatively, the particles are also detectable via detection of the plasmon signal of the particles.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0008]** The nature and mode of operation of the present invention will now be more fully described in the following detailed description of the invention taken with the accompanying drawing figure, in which:

Fig. 1 is a graph showing the reflexes detectable on the basis of the Mie scatter for specific particle diameters, namely for diameters of 20 nm, 40 nm, 60 nm, 80 nm and 100 nm, as a function of the wavelength of the illumination light.

## DETAILED DESCRIPTION OF THE INVENTION

**[0009]** According to the invention, it has been recognized that the problem occurring within the context of fluorescence microscopy is mainly attributable to the fading characteristic of the usable fluorescent die. According to the invention, there is a deviation from the marking method typically used in the biomedical field; specifically, the structures involved in the preparation are not marked with any kind of dies, but with particles having a specific diameter and specific material characteristics. While the fluorescent die attachment depends on the fluorescence behavior of the fluorescent dies assigned to the structures, the optical characteristics of the particles at some point play no role. What's more, this depends on the diameter and the material characteristics of the particles.

**[0010]** The particles are thus - insofar as required - assigned to the structures or areas of the preparations in question, it being possible to provide the particles with binding means that with certain structures can enter into a chemical bond based on adhesion. A purely mechanical bond is also conceivable. After the particles are assigned to the structure or structures involved, the structure or structures involved are detected via detection of the particles bound in or on the preparations and thus to the structures in question. In concrete terms, the structures or various areas in the preparations are differentiated in that the wavelength of the appropriate light is selected as a function of the diameter and the specific characteristics of the particles such that the particles can be detected on the basis of the Mie scatter occurring on the particles.

**[0011]** Consequently, in a manner according to the invention, a physical phenomenon that is characterized in the technical literature as "Mie scatter" is used. Reference is made here, to cite just one example, to G. Mie, *Ann. Physik* 3, 377 (1908). As far as the

theoretical reasons regarding the Mie scatter are concerned, reference is made, moreover, to P. Török et al. *"Polarized Light Microscopy"* SPIE vol. 3261, pp. 22 and following (1998). Included under the phenomenon characterized as Mie scatter or Mie reflex by the physician G. Mie is a scattering of light on particles, the scatter intensities increasing in the forward direction more strongly than in the reverse direction when the diameter of the particles grows. In contrast to the Rayleigh scatter, the Mie scatter is a function of both the material characteristics (dielectric constant, electrical conductivity) and the diameter of the scattering particles.

**[0012]** At this point it should be particularly stressed that the marking of areas or structures for their differentiated examination by assignment of particles to these structures and by subsequent detection of the particles, said particles being detected by utilization of the Mie scatter occurring on the particles. The phenomenon of the Mie scatter is thus used in a manner according to the invention to detect the particles assigned to the structures and thus to detect the structures themselves.

**[0013]** Instead of detection through Mie scatter occurring on particles, said particles can also be detected by detection of the plasmons signals. Plasmons have already been known from the literature for a long time. What is involved here is a phenomenon from the field of solid-state physics in which the electrons in the conduction band of a solid make vibrations that can be induced by, for example, light of an appropriate wavelength. This has been used primarily so far in connection with measuring devices that are based on the surface-plasmons-resonance effect. Reference is only made, for example, to US patent specification 5,351,127 in which a corresponding arrangement is described. However, for light microscopy in the classical sense, the production and detection of surface plasmons according to the previously cited publication cannot be used.

**[0014]** Within the context of the previously cited alternative detection of particles by detection of the plasmon signal, surface- or volume-plasmon resonances of the particles that are specifically tied to the structure to be detected are stimulated with appropriate light in a conventional or confocal laser scanning microscope. The surface or volume

plasmon resonances thus stimulated are then detected using suitable means. A specific detection of the various particles is possible as a function of the characteristic of the light used and the qualities of the particles used. For example there are available in spherical particles only a limited number of surface plasmons that depend on the diameter, the electron density and the dielectric characteristics of the particles.

**[0015]** Linear polarized light of a specifiable wavelength is used in an advantageous manner in order to be able to use or detect specifically the Mie effect or the Mie scatter occurring on the particles especially well. This is especially true if the wavelength of the light is larger than or approximately equal to the diameter of the particles.

**[0016]** In an especially advantageous manner, the wavelength of the light could be within the range between 300 nm and 1,500 nm. The size of particles could be below the optical resolution. In concrete terms, this means a particle diameter in the range between 10 nm and 1,000 nm is needed in order to be able to optimally use specifically the Mie scatter for the detection of the particles.

**[0017]** In a likewise advantageous manner, the wavelength of the light – for a given particle size and given specific characteristics of the particles – is selected such that a maximum Mie reflex can be detected. Starting with the theoretical reasons that are foundational here and literature references cited at the outset, reference is made on this to Figure 1 or to the graphic there, which shows the reflexes detectable on the basis of the Mie scatter for specific particle diameters, namely for diameters of 20 nm, 40 nm, 60 nm, 80 nm and 100 nm, as a function of the wavelength of the illumination light. According to this illustration, for given particle sizes those wavelengths of the light can be selected for which – for a specific particle of a given diameter – a maximum Mie reflex or a maximum Mie scatter is provable and therefore detectable.

**[0018]** It is theoretically possible that not just one area of the preparation is provided with a type of particle – of equal diameter and of equal characteristics – but rather that areas of the preparation differentiated from each other are provided with particles of varying diameter, so that the areas are simultaneously detectable via appropriate light of

various wavelengths. It is also possible to provide the areas of the preparation to be differentiated with particles of various specific characteristics – for different or equal diameters – so that the areas are simultaneously detectable via corresponding light of different or equal wavelengths. Both variants can be realized to differentiate between various areas of the preparation.

**[0019]** The particles used for marking are preferably metal particles, and specifically on the basis of their dielectric constant and electrical conductivity. The particles can also be particles metalized on the surface. The particles are furthermore configured preferably as ellipsoids or beads, specifically in order to maintain a homogeneous Mie scatter on the particles.

**[0020]** The particles can be detected via the Mie scatter or the Mie reflexes occurring there using a microscope, and specifically in both transmission microscope mode and in reflection microscope mode. If the detection occurs in transmission microscope mode, then a conventional polarization transmission microscope or a confocal polarization transmission microscope could be used. If the detection occurs in reflection microscope mode, a conventional polarization reflection microscope or a confocal polarization reflection microscope could be used to implement the detection method.

**[0021]** A high-pressure lamp, for example, is a possible light source, and it should preferably have means for selecting the wavelength and polarizing the light. This means for selecting wavelength and polarizing can also be – separately – connected in series to a traditional high-pressure lamp.

**[0022]** In an especially advantageous manner a laser can be used as a light source, especially if confocal laser scanning microscopy is to be employed. In an advantageous manner, this is a laser that emits polarized light of one wavelength. The use of a laser that emits polarized light of several different wavelengths is likewise conceivable, wavelength selecting means being connected in series – integrally or separately – to the laser. Conventional lasers and conventional wavelength-selecting means can be fallback options in this context.

**[0023]** For optimal detection of the Mie signal, it is of additional advantage if an OPO (optical parametric oscillator) connected in series with the laser is used as the light source. As a result it is specifically possible to adjust the lighting wave length almost continually, maximum detection signals being thereby detectable for a specific particle type.

**[0024]** With regard to the analysis of the preparation, it is advantageous if several image recordings are considered specifically in order to be able to eliminate or compensate for errors in picture recording. In this regard a conventional transmitted light microscopic image could also be recorded using the same microscope and taken into consideration in the image evaluation. Digital image processing methods can be used in this case. Nevertheless, systematic errors of the microscope in question can be eliminated or compensated by comparing it to a conventional transmitted light microscopic image.

**[0025]** It is also conceivable that for the analysis of the preparation on a recorded image, a conventional reflection microscopic image is recorded using the same microscope and taken into consideration in the image recording. Also in this case digital processing methods can be used. The processing of a reflection microscopic image is also conceivable, provided that both images are recorded using the same microscope. These images are used for the analysis of the preparation and considered in the image evaluation after employing digital image processing methods.

**[0026]** With regard to the image recording and subsequent image processing, it is of further advantage if several picture recordings are performed under various lighting-/detection angles. These recorded images can also be taken into consideration for image evaluation in order to be able for example to eliminate shadow effects or the like that falsify the analysis or the result. Digital processing methods can also be employed in this case.

**[0027]** The light used to detect the particles and thus for the differentiated examination of various structures can be provided via a single light source, thus, for example, via a laser light source as per the foregoing description. However, it is

necessary to provide light with several wavelengths in order to detect particles with various diameters and/or with different characteristics; thus, to this end several light sources that emit light with appropriate wavelengths simultaneously or at different times can be used. In this respect a detection of the particles assigned to the various structures that is simultaneous or at different times is possible.

**[0028]** It has already been mentioned previously that the particles on the one hand can be metallic particles and on the other hand can be particles with a metallic surface. To bind the particles to the preparation or to the structures in question, it is very advantageous if the particles are coated on the surface and if the coating enables a bond to appropriate complementary structures of the preparation. The bond can be achieved mechanically, adhesively or completely chemically.

**[0029]** Finally, it should be emphasized that the method according to the invention has the enormous advantage, compared to the traditional fluorescence microscope, that the particles used for marking – in contrast to the fluorescent dies – do not change over time and during the radiation. Furthermore, the sensors used to detect the Mie scatter or the Mie reflection must not be configured so sensitively as is the case with the fluorescent microscope – for detection of the fluorescence phenomena. If then the preparations or their structures are prepared with the particles used here, additional examinations can be reproduced on the preparations, even after considerable radiation. In any case, it is not the particles used for marking that are problematic in this context, but rather just the consistency of the preparation itself. In any case, in a manner according to the invention it is no longer necessary to be concerned about markings that change over time.

WHAT IS CLAIMED IS:

Express Mail Label No. EL917291267US LASP:111_US_

Substitute Specification - Marked Version

# TITLE OF THE INVENTION

Method for the Differentiated Examination of Various Structures in Preferably Biological Preparations

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** The present application is the U.S. national phase under 35 U.S.C. 371 of International Application No. PCT/DE99/03946 filed December 10, 1999 claiming priority of German Patent Application No. 198 58 431.8 filed December 17, 1998 and German Patent Application No. 199 50 909.3 filed October 22, 1999.

## FIELD OF THE INVENTION

**[0002]** The invention relates to a method for the differentiated examination of various structures in preferably biological preparations, in particular via confocal laser microscopy.

## BACKGROUND OF THE INVENTION

**[0003]** Fundamentally, this involves a detection/marking method, in particular a method as is applied within the context conventional fluorescence microscopy within the biomedical field. However, the fluorescence microscopy previously employed is in practice exceptionally problematic, particularly since the fluorescent dies used therein fade over time, and specifically have a fading characteristic that prevents the reproduction of examinations. Because of this fading characteristic, the fluorescence intensities change even during the course of microexamination and in particular when there is radiation of the fluorescent die with excitation light. This not only makes a reproduction of the examination impossible, but, what is more, it also makes any examination subsequent to radiation of the biological/medical preparation more difficult or – in terms of a reliable evaluation – makes such an examination nearly impossible.

## BRIEF SUMMARY OF THE INVENTION

**[0004]** The objective of the present invention is therefore to configure a method of the type in question for differentiated examination of various structures in preferably biological preparations, in particular via confocal laser scanning microscopy, such that the reproduction of the marking or of the result of the examination is ensured, even after prolonged radiation. The problems occurring in fluorescence microscopy or in connection with fluorescent die binding are to be thereby prevented.

**[0005]** This objective is met via [the features of patent claim 1] a method for the differentiated examination of various structures in preferably biological preparations, in particular using a confocal laser scanning microscope, as described and claimed herein. Accordingly, the method for differentiated examination of various structures in preferably biological preparations is characterized in that particles with a specific diameter and specific characteristics are assigned to the structures, and said structures are detected by detection of the particles specifically bound in or on the preparations.

**[0006]** In an advantageous manner the particles are detected by virtue of the wavelength of the appropriate light being selected as a function of the diameter and the specific characteristics of the particles such that the particles can be detected on the basis of the Mie scatter or Mie reflexes occurring on the particles.

**[0007]** Alternatively, the particles are also detectable via detection of the plasmon signal of the particles.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0008]** The nature and mode of operation of the present invention will now be more fully described in the following detailed description of the invention taken with the accompanying drawing figure, in which:

Fig. 1 is a graph showing the reflexes detectable on the basis of the Mie scatter for specific particle diameters, namely for diameters of 20 nm, 40 nm, 60 nm, 80 nm and 100 nm, as a function of the wavelength of the illumination light.

## DETAILED DESCRIPTION OF THE INVENTION

**[0009]** According to the invention, it has been recognized that the problem occurring within the context of fluorescence microscopy is mainly attributable to the fading characteristic of the usable fluorescent die. According to the invention, there is a deviation from the marking method typically used in the biomedical field; specifically, the structures involved in the preparation are not marked with any kind of dies, but with particles having a specific diameter and specific material characteristics. While the fluorescent die attachment depends on the fluorescence behavior of the fluorescent dies assigned to the structures, the optical characteristics of the particles at some point play no role. What's more, this depends on the diameter and the material characteristics of the particles.

**[0010]** The particles are thus - insofar as required - assigned to the structures or areas of the preparations in question, it being possible to provide the particles with binding means that with certain structures can enter into a chemical bond based on adhesion. A purely mechanical bond is also conceivable. After the particles are assigned to the structure or structures involved, the structure or structures involved are detected via detection of the particles bound in or on the preparations and thus to the structures in question. In concrete terms, the structures or various areas in the preparations are differentiated in that the wavelength of the appropriate light is selected as a function of the diameter and the specific characteristics of the particles such that the particles can be detected on the basis of the Mie scatter occurring on the particles.

**[0011]** Consequently, in a manner according to the invention, a physical phenomenon that is characterized in the technical literature as "Mie scatter" is used. Reference is made here, to cite just one example, to G. Mie, *Ann. Physik* 3, 377 (1908). As far as the

theoretical reasons regarding the Mie scatter are concerned, reference is made, moreover, to P. Török et al. *"Polarized Light Microscopy"* SPIE vol. 3261, pp. 22 and following (1998). Included under the phenomenon characterized as Mie scatter or Mie reflex by the physician G. Mie is a scattering of light on particles, the scatter intensities increasing in the forward direction more strongly than in the reverse direction when the diameter of the particles grows. In contrast to the Rayleigh scatter, the Mie scatter is a function of both the material characteristics (dielectric constant, electrical conductivity) and the diameter of the scattering particles.

**[0012]** At this point it should be particularly stressed that the marking of areas or structures for their differentiated examination by assignment of particles to these structures and by subsequent detection of the particles, said particles being detected by utilization of the Mie scatter occurring on the particles. The phenomenon of the Mie scatter is thus used in a manner according to the invention to detect the particles assigned to the structures and thus to detect the structures themselves.

**[0013]** Instead of detection through Mie scatter occurring on particles, said particles can also be detected by detection of the plasmons signals. Plasmons have already been known from the literature for a long time. What is involved here is a phenomenon from the field of solid-state physics in which the electrons in the conduction band of a solid make vibrations that can be induced by, for example, light of an appropriate wavelength. This has been used primarily so far in connection with measuring devices that are based on the surface-plasmons-resonance effect. Reference is only made, for example, to US patent specification 5,351,127 in which a corresponding arrangement is described. However, for light microscopy in the classical sense, the production and detection of surface plasmons according to the previously cited publication cannot be used.

**[0014]** Within the context of the previously cited alternative detection of particles by detection of the plasmon signal, surface- or volume-plasmon resonances of the particles that are specifically tied to the structure to be detected are stimulated with appropriate light in a conventional or confocal laser scanning microscope. The surface or volume

plasmon resonances thus stimulated are then detected using suitable means. A specific detection of the various particles is possible as a function of the characteristic of the light used and the qualities of the particles used. For example there are available in spherical particles only a limited number of surface plasmons that depend on the diameter, the electron density and the dielectric characteristics of the particles.

**[0015]** Linear polarized light of a specifiable wavelength is used in an advantageous manner in order to be able to use or detect specifically the Mie effect or the Mie scatter occurring on the particles especially well. This is especially true if the wavelength of the light is larger than or approximately equal to the diameter of the particles.

**[0016]** In an especially advantageous manner, the wavelength of the light could be within the range between 300 nm and 1,500 nm. The size of particles could be below the optical resolution. In concrete terms, this means a particle diameter in the range between 10 nm and 1,000 nm is needed in order to be able to optimally use specifically the Mie scatter for the detection of the particles.

**[0017]** In a likewise advantageous manner, the wavelength of the light – for a given particle size and given specific characteristics of the particles – is selected such that a maximum Mie reflex can be detected. Starting with the theoretical reasons that are foundational here and literature references cited at the outset, reference is made on this to Figure 1 or to the graphic there, which shows the reflexes detectable on the basis of the Mie scatter for specific particle diameters, namely for diameters of 20 nm, 40 nm, 60 nm, 80 nm and 100 nm, as a function of the wavelength of the illumination light. According to this illustration, for given particle sizes those wavelengths of the light can be selected for which – for a specific particle of a given diameter – a maximum Mie reflex or a maximum Mie scatter is provable and therefore detectable.

**[0018]** It is theoretically possible that not just one area of the preparation is provided with a type of particle – of equal diameter and of equal characteristics – but rather that areas of the preparation differentiated from each other are provided with particles of varying diameter, so that the areas are simultaneously detectable via appropriate light of

various wavelengths. It is also possible to provide the areas of the preparation to be differentiated with particles of various specific characteristics – for different or equal diameters – so that the areas are simultaneously detectable via corresponding light of different or equal wavelengths. Both variants can be realized to differentiate between various areas of the preparation.

**[0019]** The particles used for marking are preferably metal particles, and specifically on the basis of their dielectric constant and electrical conductivity. The particles can also be particles metalized on the surface. The particles are furthermore configured preferably as ellipsoids or beads, specifically in order to maintain a homogeneous Mie scatter on the particles.

**[0020]** The particles can be detected via the Mie scatter or the Mie reflexes occurring there using a microscope, and specifically in both transmission microscope mode and in reflection microscope mode. If the detection occurs in transmission microscope mode, then a conventional polarization transmission microscope or a confocal polarization transmission microscope could be used. If the detection occurs in reflection microscope mode, a conventional polarization reflection microscope or a confocal polarization reflection microscope could be used to implement the detection method.

**[0021]** A high-pressure lamp, for example, is a possible light source, and it should preferably have means for selecting the wavelength and polarizing the light. This means for selecting wavelength and polarizing can also be – separately – connected in series to a traditional high-pressure lamp.

**[0022]** In an especially advantageous manner a laser can be used as a light source, especially if confocal laser scanning microscopy is to be employed. In an advantageous manner, this is a laser that emits polarized light of one wavelength. The use of a laser that emits polarized light of several different wavelengths is likewise conceivable, wavelength selecting means being connected in series – integrally or separately – to the laser. Conventional lasers and conventional wavelength-selecting means can be fallback options in this context.

**[0023]** For optimal detection of the Mie signal, it is of additional advantage if an OPO (optical parametric oscillator) connected in series with the laser is used as the light source. As a result it is specifically possible to adjust the lighting wave length almost continually, maximum detection signals being thereby detectable for a specific particle type.

**[0024]** With regard to the analysis of the preparation, it is advantageous if several image recordings are considered specifically in order to be able to eliminate or compensate for errors in picture recording. In this regard a conventional transmitted light microscopic image could also be recorded using the same microscope and taken into consideration in the image evaluation. Digital image processing methods can be used in this case. Nevertheless, systematic errors of the microscope in question can be eliminated or compensated by comparing it to a conventional transmitted light microscopic image.

**[0025]** It is also conceivable that for the analysis of the preparation on a recorded image, a conventional reflection microscopic image is recorded using the same microscope and taken into consideration in the image recording. Also in this case digital processing methods can be used. The processing of a reflection microscopic image is also conceivable, provided that both images are recorded using the same microscope. These images are used for the analysis of the preparation and considered in the image evaluation after employing digital image processing methods.

**[0026]** With regard to the image recording and subsequent image processing, it is of further advantage if several picture recordings are performed under various lighting-/detection angles. These recorded images can also be taken into consideration for image evaluation in order to be able for example to eliminate shadow effects or the like that falsify the analysis or the result. Digital processing methods can also be employed in this case.

**[0027]** The light used to detect the particles and thus for the differentiated examination of various structures can be provided via a single light source, thus, for example, via a laser light source as per the foregoing description. However, it is

necessary to provide light with several wavelengths in order to detect particles with various diameters and/or with different characteristics; thus, to this end several light sources that emit light with appropriate wavelengths simultaneously or at different times can be used. In this respect a detection of the particles assigned to the various structures that is simultaneous or at different times is possible.

**[0028]** It has already been mentioned previously that the particles on the one hand can be metallic particles and on the other hand can be particles with a metallic surface. To bind the particles to the preparation or to the structures in question, it is very advantageous if the particles are coated on the surface and if the coating enables a bond to appropriate complementary structures of the preparation. The bond can be achieved mechanically, adhesively or completely chemically.

**[0029]** Finally, it should be emphasized that the method according to the invention has the enormous advantage, compared to the traditional fluorescence microscope, that the particles used for marking – in contrast to the fluorescent dies – do not change over time and during the radiation. Furthermore, the sensors used to detect the Mie scatter or the Mie reflection must not be configured so sensitively as is the case with the fluorescent microscope – for detection of the fluorescence phenomena. If then the preparations or their structures are prepared with the particles used here, additional examinations can be reproduced on the preparations, even after considerable radiation. In any case, it is not the particles used for marking that are problematic in this context, but rather just the consistency of the preparation itself. In any case, in a manner according to the invention it is no longer necessary to be concerned about markings that change over time.

WHAT IS CLAIMED IS: [Patent claims]

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# Method for the Differentiated Examination of Various Structures in Preferably Biological Preparations

The invention relates to a method for the differentiated examination of various structures in preferably biological preparations, in particular via confocal laser microscopy.

Fundamentally, this involves a detection/marking method, in particular a method as is applied within the context conventional fluorescence microscopy within the biomedical field. However, the fluorescence microscopy previously employed is in practice exceptionally problematic, particularly since the fluorescent dies used therein fade over time, and specifically have a fading characteristic that prevents the reproduction of examinations. Because of this fading characteristic, the fluorescence intensities change even during the course of microexamination and in particular when there is radiation of the fluorescent die with excitation light. This not only makes a reproduction of the examination impossible, but, what is more, it also makes any examination subsequent to radiation of the biological/medical preparation more difficult or – in terms of a reliable evaluation – makes such an examination nearly impossible.

The objective of the present invention is therefore to configure a method of the type in question for differentiated examination of various structures in preferably biological preparations, in particular via confocal laser scanning microscopy, such that the reproduction of the marking or of the result of the examination is ensured, even after prolonged radiation. The problems occurring in fluorescence microscopy or in connection with fluorescent die binding are to be thereby prevented.

This objective is met via the features of patent claim 1. Accordingly, the method for differentiated examination of various structures in preferably biological preparations is

characterized in that particles with a specific diameter and specific characteristics are assigned to the structures, and said structures are detected by detection of the particles specifically bound in or on the preparations.

In an advantageous manner the particles are detected by virtue of the wavelength of the appropriate light being selected as a function of the diameter and the specific characteristics of the particles such that the particles can be detected on the basis of the Mie scatter or Mie reflexes occurring on the particles.

Alternatively, the particles are also detectable via detection of the plasmon signal of the particles.

According to the invention, it has been recognized that the problem occurring within the context of fluorescence microscopy is mainly attributable to the fading characteristic of the usable fluorescent die. According to the invention, there is a deviation from the marking method typically used in the biomedical field; specifically, the structures involved in the preparation are not marked with any kind of dies, but with particles having a specific diameter and specific material characteristics. While the fluorescent die attachment depends on the fluorescence behavior of the fluorescent dies assigned to the structures, the optical characteristics of the particles at some point play no role. What's more, this depends on the diameter and the material characteristics of the particles.

The particles are thus - insofar as required - assigned to the structures or areas of the preparations in question, it being possible to provide the particles with binding means that with certain structures can enter into a chemical bond based on adhesion. A purely mechanical bond is also conceivable. After the particles are assigned to the structure or

structures involved, the structure or structures involved are detected via detection of the particles bound in or on the preparations and thus to the structures in question. In concrete terms, the structures or various areas in the preparations are differentiated in that the wavelength of the appropriate light is selected as a function of the diameter and the specific characteristics of the particles such that the particles can be detected on the basis of the Mie scatter occurring on the particles.

Consequently, in a manner according to the invention, a physical phenomenon that is characterized in the technical literature as "Mie scatter" is used. Reference is made here, to cite just one example, to G. Mie, *Ann. Physik* 3, 377 (1908). As far as the theoretical reasons regarding the Mie scatter are concerned, reference is made, moreover, to P. Török et al. *"Polarized Light Microscopy"* SPIE vol. 3261, pp. 22 and following (1998). Included under the phenomenon characterized as Mie scatter or Mie reflex by the physician G. Mie is a scattering of light on particles, the scatter intensities increasing in the forward direction more strongly than in the reverse direction when the diameter of the particles grows. In contrast to the Rayleigh scatter, the Mie scatter is a function of both the material characteristics (dielectric constant, electrical conductivity) and the diameter of the scattering particles.

At this point it should be particularly stressed that the marking of areas or structures for their differentiated examination by assignment of particles to these structures and by subsequent detection of the particles, said particles being detected by utilization of the Mie scatter occurring on the particles. The phenomenon of the Mie scatter is thus used in a manner according to the invention to detect the particles assigned to the structures and thus to detect the structures themselves.

Instead of detection through Mie scatter occurring on particles, said particles can also be detected by detection of the plasmons signals. Plasmons have already been known from the literature for a long time. What is involved here is a phenomenon from the field of solid-state physics in which the electrons in the conduction band of a solid make vibrations that can be induced by, for example, light of an appropriate wavelength. This has been used primarily so far in connection with measuring devices that are based on the surface-plasmons-resonance effect. Reference is only made, for example, to US patent specification 5,351,127 in which a corresponding arrangement is described. However, for light microscopy in the classical sense, the production and detection of surface plasmons according to the previously cited publication cannot be used.

Within the context of the previously cited alternative detection of particles by detection of the plasmon signal, surface- or volume-plasmon resonances of the particles that are specifically tied to the structure to be detected are stimulated with appropriate light in a conventional or confocal laser scanning microscope. The surface or volume plasmon resonances thus stimulated are then detected using suitable means. A specific detection of the various particles is possible as a function of the characteristic of the light used and the qualities of the particles used. For example there are available in spherical particles only a limited number of surface plasmons that depend on the diameter, the electron density and the dielectric characteristics of the particles.

Linear polarized light of a specifiable wavelength is used in an advantageous manner in order to be able to use or detect specifically the Mie effect or the Mie scatter occurring on the particles especially well. This is especially true if the wavelength of the light is larger than or approximately equal to the diameter of the particles.

In an especially advantageous manner, the wavelength of the light could be within the range between 300 nm and 1,500 nm. The size of particles could be below the optical resolution. In concrete terms, this means a particle diameter in the range between 10 nm and 1,000 nm is needed in order to be able to optimally use specifically the Mie scatter for the detection of the particles.

In a likewise advantageous manner, the wavelength of the light – for a given particle size and given specific characteristics of the particles – is selected such that a maximum Mie reflex can be detected. Starting with the theoretical reasons that are foundational here and literature references cited at the outset, reference is made on this to Figure 1 or to the graphic there, which shows the reflexes detectable on the basis of the Mie scatter for specific particle diameters, namely for diameters of 20 nm, 40 nm, 60 nm, 80 nm and 100 nm, as a function of the wavelength of the illumination light. According to this illustration, for given particle sizes those wavelengths of the light can be selected for which – for a specific particle of a given diameter – a maximum Mie reflex or a maximum Mie scatter is provable and therefore detectable.

It is theoretically possible that not just one area of the preparation is provided with a type of particle – of equal diameter and of equal characteristics – but rather that areas of the preparation differentiated from each other are provided with particles of varying diameter, so that the areas are simultaneously detectable via appropriate light of various wavelengths. It is also possible to provide the areas of the preparation to be differentiated with particles of various specific characteristics – for different or equal diameters – so that the areas are simultaneously detectable via corresponding light of different or equal wavelengths. Both variants can be realized to differentiate between various areas of the preparation.

The particles used for marking are preferably metal particles, and specifically on the basis of their dielectric constant and electrical conductivity. The particles can also be particles metalized on the surface. The particles are furthermore configured preferably as ellipsoids or beads, specifically in order to maintain a homogeneous Mie scatter on the particles.

The particles can be detected via the Mie scatter or the Mie reflexes occurring there using a microscope, and specifically in both transmission microscope mode and in reflection microscope mode. If the detection occurs in transmission microscope mode, then a conventional polarization transmission microscope or a confocal polarization transmission microscope could be used. If the detection occurs in reflection microscope mode, a conventional polarization reflection microscope or a confocal polarization reflection microscope could be used to implement the detection method.

A high-pressure lamp, for example, is a possible light source, and it should preferably have means for selecting the wavelength and polarizing the light. This means for selecting wavelength and polarizing can also be – separately – connected in series to a traditional high-pressure lamp.

In an especially advantageous manner a laser can be used as a light source, especially if confocal laser scanning microscopy is to be employed. In an advantageous manner, this is a laser that emits polarized light of one wavelength. The use of a laser that emits polarized light of several different wavelengths is likewise conceivable, wavelength selecting means being connected in series – integrally or separately – to the laser. Conventional lasers and conventional wavelength-selecting means can be fallback options in this context.

For optimal detection of the Mie signal, it is of additional advantage if an OPO (optical parametric oscillator) connected in series with the laser is used as the light source. As a result it is specifically possible to adjust the lighting wave length almost continually, maximum detection signals being thereby detectable for a specific particle type.

With regard to the analysis of the preparation, it is advantageous if several image recordings are considered specifically in order to be able to eliminate or compensate for errors in picture recording. In this regard a conventional transmitted light microscopic image could also be recorded using the same microscope and taken into consideration in the image evaluation. Digital image processing methods can be used. in this case. Nevertheless, systematic errors of the microscope in question can be eliminated or compensated by comparing it to a conventional transmitted light microscopic image.

It is also conceivable that for the analysis of the preparation on a recorded image, a conventional reflection microscopic image is recorded using the same microscope and taken into consideration in the image recording. Also in this case digital processing methods can be used. The processing of a reflection microscopic image is also conceivable, provided that both images are recorded using the same microscope. These images are used for the analysis of the preparation and considered in the image evaluation after employing digital image processing methods.

With regard to the image recording and subsequent image processing, it is of further advantage if several picture recordings are performed under various lighting-/detection angles. These recorded images can also be taken into consideration for image evaluation

in order to be able for example to eliminate shadow effects or the like that falsify the analysis or the result. Digital processing methods can also be employed in this case.

The light used to detect the particles and thus for the differentiated examination of various structures can be provided via a single light source, thus, for example, via a laser light source as per the foregoing description. However, it is necessary to provide light with several wavelengths in order to detect particles with various diameters and/or with different characteristics; thus, to this end several light sources that emit light with appropriate wavelengths simultaneously or at different times can be used. In this respect a detection of the particles assigned to the various structures that is simultaneous or at different times is possible.

It has already been mentioned previously that the particles on the one hand can be metallic particles and on the other hand can be particles with a metallic surface. To bind the particles to the preparation or to the structures in question, it is very advantageous if the particles are coated on the surface and if the coating enables a bond to appropriate complementary structures of the preparation. The bond can be achieved mechanically, adhesively or completely chemically.

Finally, it should be emphasized that the method according to the invention has the enormous advantage, compared to the traditional fluorescence microscope, that the particles used for marking – in contrast to the fluorescent dies – do not change over time and during the radiation. Furthermore, the sensors used to detect the Mie scatter or the Mie reflection must not be configured so sensitively as is the case with the fluorescent microscope – for detection of the fluorescence phenomena. If then the preparations or their structures are prepared with the particles used here, additional examinations can be

reproduced on the preparations, even after considerable radiation. In any case, it is not the particles used for marking that are problematic in this context, but rather just the consistency of the preparation itself. In any case, in a manner according to the invention it is no longer necessary to be concerned about markings that change over time.

5

Patent claims

1. A method for the differentiated examination of various structures in preferably biological preparations, in particular using a confocal laser scanning microscope, wherein particles with specific diameter and specific qualities are assigned to the structures and the structures are detected by detection of the particles specifically bound in or on the preparations.

2. The method as recited in Claim 1, wherein the detection of the particles occurs by the wavelength of suitable light being selected as a function of the diameter and of the specific characteristics of the particles such that said particles can be detected on the basis of the Mie scatter occurring on the particles.

3. The method as recited in Claim 1, wherein the particles are detected by the wavelength of a suitable light being selected as a function of the diameter and of the specific characteristics of the particles such that said particles can be detected on the basis of the plasmon signal occurring on the particles.

4. The method as recited in any of Claims 1 through 3, wherein linear polarized light of specifiable wavelength is used.

5. The method as recited in any of Claims 1 through 4, wherein the wavelength of the light is larger than or is somewhat equal to the diameter of the particles.

6. The method as recited in any of Claims 1 through 5, wherein the wavelength of the light is within a range between 300 nm and 1,500 nm.

7. The method as recited in any of Claims 1 through 6, wherein the size of the particles is below the optical resolution.

8. The method as recited in any of Claims 1 through 7, wherein the particles have a diameter within the range between 10 nm and 1,500 nm.

9. The method as recited in any of Claims 1 through 8, wherein the wavelength of the light is selected such that a maximum Mie reflex is detectable for a given particle size and specific characteristic of the particles.

10. The method as recited in any of Claims 1 through 9, wherein areas of the preparation to be differentiated are provided with particles of various diameters, so that the differentiating areas are detected simultaneously or successively by means of suitable light of various wavelengths.

11. The method as recited in any of Claims 1 through 10, wherein areas of the preparation to be differentiated are provided with particles of specific characteristics, so that the differentiating areas are detected simultaneously or successively by means of suitable light of varying or equal wavelengths.

12. The method as recited in any of Claims 1 through 11, wherein the particles are metal particles.

13. The method as recited in any of Claims 1 through 12, wherein the particles are particles that are metalized on the surface.

14. The method as recited in any of Claims 1 through 13, wherein the particles are formed as ellipsoids or beads.

15. The method as recited in any of Claims 1 through 14, wherein the particles are detected via the Mie-reflexes occurring there in transmission microscope mode.

16. The method as recited in Claim 15, wherein the microscope used is a conventional polarization transmission microscope.

17. The method as recited in Claim 16, wherein the microscope used is a confocal polarization transmission microscope.

18. The method as recited in Claim 1 and 14, wherein the particles are specifically detected via the Mie-reflexes occurring there in the reflection microscope mode.

19. The method as recited in Claim 18, wherein the microscope used is a conventional polarization reflection microscope.

20. The method as recited in Claim 19, wherein the microscope used is a confocal polarization reflection microscope.

21. The method as recited in any of Claims 1 through 20, wherein the light is produced using a high-pressure lamp as the light source, preferably with means for wavelength selection and polarization.

22. The method as recited in any of Claims 1 through 20, wherein a laser that emits polarized light of one wavelength is used as the light source.

23. The method as recited in any of Claims 1 through 20, wherein the light is produced using an OPO (optical parametric oscillator) as the light source, with which the wavelength of the illuminating light can be varied, with the goal of being able to measure a maximum Mie-signal for a specific particle type.

24. The method as recited in any of Claims 1 through 20, wherein a laser that emits polarized light of several different wavelengths is used as the light source, means for selecting wavelengths being connected in series - integrally or separately - to the laser.

25. The method as recited in any of Claims 1 through 24, wherein to analyze biological preparations on a recorded image, in addition to using the same microscope, a conventional transmitted light microscopic image is recorded and is taken into consideration in the image evaluation using, for example, digital image processing methods.

26. The method as recited in any of Claims 1 through 24, wherein to analyze biological preparations on a recorded image, in addition to using the same microscope, a conventional reflection microscopic image is recorded and is taken into consideration in the image evaluation using, for example, digital image processing methods.

27. The method as recited in any of Claims 1 through 26, wherein both a reflection microscopic image and a transmitted light microscopic image of the preparation is recorded using the same microscope, used for analysis of the preparation and is taken into consideration in the image evaluation using, for example, digital image processing methods.

28. The method as recited in any of Claims 1 through 27, wherein image recordings are carried out under various lighting/detection angles, and these image acquisitions are taken into consideration in the image evaluation using, for example, digital image processing methods.

29. The method as recited in any of Claims 1 through 28, wherein the light used for detection of the particles is provided via a single light source.

30. The method as recited in any of Claims 1 through 29, wherein the light used for detection of the particles having various diameters and/ various characteristics is provided simultaneously or at different times via a multiplicity of light sources of appropriate wavelength.

31. The method as recited in any of Claims 1 through 30, wherein the particles are coated on the surface and the coating enables a specific bonding to corresponding complementary structures of the preparation.

ABSTRACT

The invention relates to a method for examining different structures in preferably biological preparations in a differential manner, especially by means of confocal laser scanning microscopy. The method is characterized in that particles having a specific diameter and specific characteristics are assigned to the structures and in that said structures are detected by detecting the particles which have specifically bonded in or to the preparations. The detection process is carried out in an advantageous manner by marking the structures with metal particles with diameters of 10 nm to 1,500 nm and detecting Mie scattering or a plasmon signal.

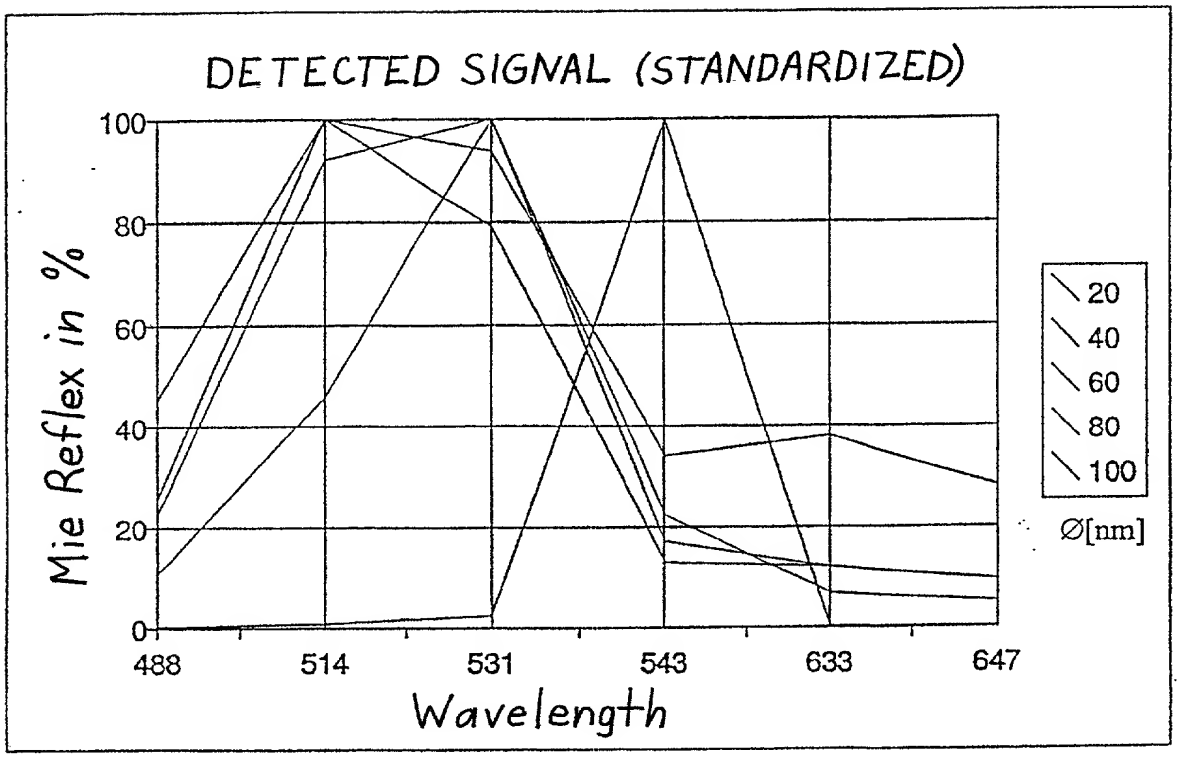
DETECTED SIGNAL (STANDARDIZED)
Mie Reflex in %
100
80
60
40
20
0
488
514
531
543
633
647
Wavelength
20
40
60
80
100
Ø[nm]

Fig. 1

| Prior Foreign Applications<br>(Frühere ausländische Anmeldungen) | | | Priority Not Claimed<br>(Priorität nicht beansprucht) |
|---|---|---|---|
| **198 58 431.8** | **Germany** | **17/December/1998** ✓ | ☐ |
| **199 50 909.3** | **Germany** | **22/October/1999** ✓ | ☐ |
| App. No. | Country | Day/Month/Year | ☐ |

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App. No. Filed: Status: Patented/Pending/Abandoned

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## German Language Declaration

Als nachstehend benannter Erfinder erkläre ich hiermit an Eides Statt:

daß mein Wohnsitz meine Postanschrift und meine Staatsangehörigkeit den im nachstehenden nach meinem Namen aufgeführten Angaben entsprechen, daß ich nach bestem Wissen der ursprüngliche, erste und alleinige Erfinder (falls nachstehend nur ein Name angegeben ist) odor ein ursprünglicher, erster und Miterfinder (falls nachstehend mehrere Namen aufgeführt sind) des Gegenstandes bin, für den dieser Antrag gestellt wird und für den ein Patent für die Erfindung mit folgendem Titel beantragt wird:

**VERFAHREN ZUR DIFFERENZIERTEN UNTERSUSCHUNG UNTERSCHIEDLICHER STRUKTUREN IN VORZUGSWEISE BIOLOGISCHEN PRÄPARATEN**

deren Beschreibung hier beigefügt ist, es sei denn (in diesem Falle Zutreffendes bitte ankreuzen), diese Erfindung

☒ wurde angemeldet am **10-December-1999** unter der US-Anmeldenummer oder unter der Internationalen Anmeldenummer im Rahmen des Vertrags über die Zusammenarbeit auf dem Gebiet des Patentwesens (PCT) **PCT/DE99/03946** und am _____ abgeändert (falls zutreffend).

Ich bestätige hiermit, daß ich den Inhalt der oben angegebenen Patentanmeldung, einschließlich der Ansprüche, die eventuell durch einen oben erwähnten Zusatzantrag abgeändert wurde, durchgesehen und verstanden habe.

Ich erkenne meine Pflicht zur Offenbarung jeglicher Informationen an, die zur Prüfung der Patentfähigkeit in Einklang mit Titel 37, Code of Federal Regulations, 1.56 von Belang sind.

Ich beanspruche hiermit ausländische Prioritätsvorteile gemäß Title 35, US Code, 119(a)-(d), bzw. 365(b) aller unten aufgeführten Auslandsanmeldungen für Patente oder Erfinderurkunden, oder 365(a) aller PCT internationalen Anmeldungen, welche wenigstens ein Land ausser den Vereinigten Staaten von Amerika benennen, und habe nachstehend durch ankreuzen sämtliche Auslands- anmeldungen für Patente bzw. Erfinderurkunden oder PCT internatioonale Anmeldungen angegeben, deren Anmeldetag dem der Anmeldung, für welche Priorität beansprucht wird, vorangeht.

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHOD FOR DIFFERENTIATED INVESTIGATION OF DIVERSE STRUCTURES IN PREFERABLY BIOLOGICAL PREPARATIONS**

the specification of which is attached hereto unless the following box is checked:

☒ was filed on **10-December-1999** as United States Application Number or PCT International Application Number **PCT/DE99/03946** and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

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